Botanical fungicides: natural and semi-synthetic ceveratrum alkaloids†

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Abstract: The practical potential of the main bioactive principles of sabadilla, a botanical insecticide prepared from the seeds of *Schoenocaulon officinale*, and related semi-synthetic ceveratrum alkaloids in controlling phytopathogenic bacteria and fungi was explored. Evaluating 35 compounds belonging to this group of steroid alkaloids revealed antimicrobial effects against *Erwinia*, *Corynebacterium*, *Fusarium*, *Glomerella* and *Plasmopara* species. Significant activities were displayed against the sunflower downy mildew fungus, *Plasmopara halstedii*. The ceveratrum alkaloids are thought to interact with the cell membrane, disrupting its integrity and/or altering its function. In contrast to commercial fungicides affecting either the parasitising thallus or the asexual spores of *P. halstedii*, ie, only a single developmental stage, the alkaloids tested disrupt its life cycle at several points in host-dependent and host-independent stages. Among the natural alkaloids, cevacine and cevadine were the most effective against sunflower downy mildew and the activity of the semi-synthetic oleoyl veracevine and cevagenine was comparable to that of metalaxyl. Sunflowers showed good tolerance to these highly active natural and semi-synthetic ceveratrum alkaloids. Our results indicate that the modest anti-oomycete activity of the natural sabadilla components can be greatly improved by appropriate structural modifications and thus establish the ceveratrum alkaloids as potential antifungal agents with a novel mode of action.

Keywords: fungicides; sabadilla; steroid alkaloids; microbicides; *Plasmopara; Erwinia; Fusarium;* sunflower downy mildew

1 INTRODUCTION

Sabadilla powder, prepared from the seeds of *Schoenocaulon officinale* A Gray, was once an important botanical insecticide[11] but its use is currently restricted to selective thrips control in citrus and avocado.[2,3] The main insecticidal principles of sabadilla, cevacine (**2**), cevadine (**3**), 3-*O*-vanilloylveracevine (**4**) and veratridine (**5**), are ester derivatives of the steroid alkaloid veracevine (**1**) (Table 1). These compounds belong to the ceveratrum group of *Veratrum* alkaloids[4,5] characterised by a highly hydroxylated C-nor-D-homosteroid skeleton (Fig 1).[6,7] *Veratrum* alkaloid preparations, though possessing a narrow therapeutic index, have also been used as antihypertensive agents.[8,9] These alkaloids act by stabilising the open state of the Na^+ channel of excitable cell membranes.[10,11]

Although the possible ecological role of alkaloids in plant–microbe interactions has been suggested,[12,13] and selective antifungal activity of *Veratrum* alkaloids was indicated by Wolters[14] against some fungi *in vitro*, the antimicrobial properties of ceveratrum alkaloids have not been investigated in detail. A recent structure–insecticidal activity relationship study has demonstrated that modifications of the acyl group (RCO in Fig 1) profoundly influence the selective toxicity of veracevine derivatives.[15] These results prompted us to examine the toxicological profile of this type of compound against other systematic groups.

Here we report results of comparative studies on the antimicrobial properties of sabadilla components and related derivatives, a total of 35 compounds, against 23 bacterial and 27 fungal species. The developmental stage-dependent toxicity of these compounds to *Plasmopara halstedii* (Farl) Berl & de Toni (Oomycota, Peronosporales), the causative agent of sunflower downy mildew (SDM) that exhibited particular sensitivity to ceveratrum alkaloids, is also reported.

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No	Compound[a]	Calculated log P for R-OMe[b]
	Natural alkaloids, R	
1	H (veracevine)	
2	MeCO (cevacine)	−0.14
3	(*Z*)-MeCH=(Me)CO	1.25
4	3-MeO-4-HOPhCO	1.24
5	3,4-$(MeO)_2$PhCO	1.27
	Semisynthetic derivatives, R	
6	H[c] (cevine)	na
7	Me	na
8	*i*-PrCO	1.05
9	*c*-PrCO	0.55
10	*t*-BuCO	1.72
11	(±)-$MeCH_2CH(Me)CO$	1.45
12	(*E*)-MeCH=C(Me)CO	1.25
13	(*Z*)-$Me(CH_2)_7C=C(CH_2)_7CO$	5.38
14	Et_2NCH_2CO	0.42
15	PhCO	1.78
16	4-HC≡CPhCO	1.96
17	3-MeOPhCO	1.52
18	3,5-$(MeO)_2$PhCO	1.27
19	4-$PhCH_2O$,3-MeOPhCO	3.05
20	3-PhOPhCO	3.21
21	3-CF_3CH_2O,5-MeOPhCO	2.25
22	(2-MeNHPh)CO	1.35
23	4-ClPhCO	2.29
24	3,4-Cl_2PhCO	2.81
25	3,5-I_2PhCO	4.29
26	3-ThienylCO	1.30
27	5-MeO-2-thienylCO	0.76
28	2-NaphthylCO	2.78
29	1-NaphthylCO	2.78
30	i-PrOCO	1.24
	Others	
31	Cevagenine	na
32	Cevadine-12,14,17-orthoacetate	na
33	Veratridine *N*-methiodide	na
34	4,16-Diacetylveratridine	na
35	Germine	na

[a] For general structures, see Fig 1.
[b] Lipophilicity for R-OMe esters **2–5** and **8–30** was calculated by the *ChemPlus* module of *HyperChem* 5.0 program; na = not applicable.
[c] 3-α-OH isomer.

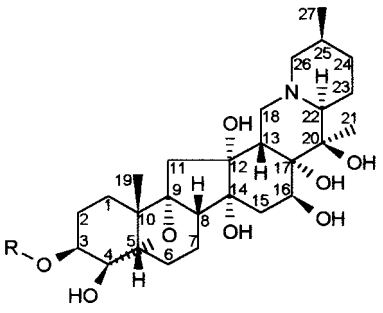
Table 1. Natural ceveratrum alkaloids and semisynthetic derivatives tested.

2 MATERIALS AND METHODS

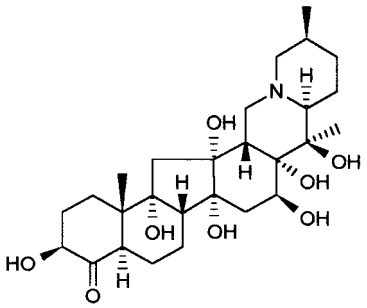
2.1 General

Test compounds **1–3, 5, 6, 8–18, 20, 22–24, 28–30,** and **32–34** were obtained as described previously.[15] 3-*O*-Vanilloylveracevine (**4**) and compound **19** were prepared according to Ujváry and Casida.[16] Cevagenine (**31**) was obtained from veratrine mixture (Sigma, St. Louis, USA) by mild alkaline hydrolysis according to literature methods.[17] Germine (**35**) was a gift from the late Prof DHR Barton (Texas A & M University, College Station, Texas). The synthesis of compounds **7**, **21** and **25–27,** which are new, is described in Section 2.2.

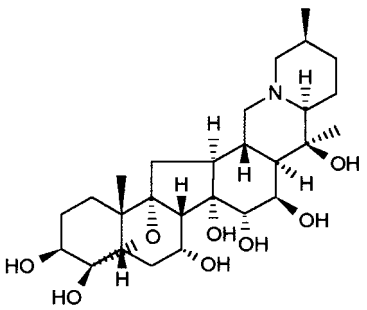
Analytical and preparative TLC were carried out on 0.25 and 2 mm silica gel plates, respectively, using cyclohexane + ethyl acetate + diethylamine (7 + 2 + 1, by volume) for development unless otherwise noted. Visualisation was by UV light (254 nm) and by dipping the plate into a solution of vanillin in ethanol (30 g litre^{-1}) containing sulfuric acid (10 ml litre^{-1}) then heating with a heat-gun. Compounds from preparative TLC were recovered by scraping the appropriate band and elution with chloroform + methanol (4 + 1, by volume). Melting points are uncorrected. [^{1}H] and [^{13}C]NMR spectra were obtained at 300 and 75 MHz, respectively, on a Bruker WM-300 spectrometer using deuterochloroform as the solvent and tetramethylsilane as the internal standard. Assignment of the carbon atoms in the [^{13}C]NMR spectra was based on previous studies with sabadilla alkaloids.[15,18] High-resolution fast atom bombardment spectrum of compound **7** was obtained with a Kratos MS-50 instrument.



1 - 30



31 (cevagenine)



35 (germine)

Figure 1. Structures of veracevine (**1**, R = H), its 3-*O*-derivatives **2–30** and related ceveratrum alkaloids.

Metalaxyl, metconazole and tridemorph were isolated from commercial 'Ridomil' 25 WP (Ciba-Geigy, Basel, Switzerland), 'Caramba' (American Cyanamid, Princeton, USA) and 'Calixin' 75 EC (BASF, Ludwigshafen, Germany), respectively. Polymyxin B sulfate was bought from Pfizer (New York, USA), streptomycin sesquisulfate was a gift of Biogal (Debrecen, Hungary), dimethomorph, dodine, nystatin and blasticidin-S were provided by Shell (Ely, UK), Agrokémia (Selye, Hungary), Chinoin (Budapest, Hungary) and Kumiai (Tokyo, Japan), respectively.

Analytical grade mineral salts, glucose, glycerol and amino acids (Reanal, Budapest, Hungary), and vitamins (Chinoin), Na-β-glycerophosphate (Sigma) and bacteriological agar No. 1, Bacto Peptone (L37), Yeast Extract (L21), Trypton T (L43) (Oxoid, Basingstoke, UK) and malt extract broth (MEB) (Sigma) were used for preparing media.

The lipophilicity of the RCO-acyl group of ester alkaloids was characterised by calculating the octanol-water partition coefficient (log *P*) for its methyl ester (Table 1). The calculation was done using an atomic parameter-based method of the ChemPlus ver. 1.6 module of the PC-based HyperChem 5.0 program (Hypercube, Inc., Gainesville, Florida, USA).

2.2 Synthesis of new compounds

2.2.1 3-O-Methylveracevine (7)

For the tertiary amine-containing compound **1**, the Lewis-acid-catalysed etherification method[19] was used. Thus, ethereal diazomethane (1 ml, *c* 0.9 mmol) was added in several portions to a solution of **1** (20 mg, 0.039 mmol) in dichloromethane containing fluoboric acid (50% by volume; 4 µl) over 10 min. The solution was stirred at room temperature for 2 h, and then partitioned between chloroform and aqueous ammonium hydroxide (10%, by volume) (25 + 10 ml), the phases were separated and the aqueous layer then extracted with chloroform (2 × 25 ml). The organic phases were combined, washed with water, dried ($MgSO_4$), filtered and concentrated. The residue was purified by pipette column chromatography (silica gel 60, 0.040–0.063 mm) using cyclohexane + ethyl acetate + diethylamine (8.5 + 1.0 + 0.5, by volume) to give the title compound as a white solid (10 mg, 49%), mp 125–127°C. [^{1}H]NMR (diagnostic peaks listed only) δ: 4.74 (1H, d, J = 5.7 Hz, H-3), 4.70 (broad s, OHs), 4.19 (1H, t, J = 3.0 Hz, H-16), 3.38 (3H, s, CH_3O), 1.15 (3H, s, H-21), 1.09 (3H, d, J = 7.0 Hz, H-27), 0.97 (3H, s, H-19). [^{13}C]NMR δ: 105.9 (C-4), 94.1 (C-9), 83.0 (C-3), 81.7 (C-12), 80.4 (C-14), 75.7 (C-20), 72.0 (C-17), 70.9 (C-16), 63.5 (C-22), 61.2 (C-26), 57.8 (CH_3O), 51.3 (C-18), 45.5 (C-10), 44.9 (C-5), 44.5 (C-8), 42.0 (C-11), 36.9 (C-13), 32.0 (C-1), 31.1 (C-15), 29.0 (C-23), 27.6 (C-6), 27.5 (C-25), 19.2 (C-19), 18.7 (C-24), 18.3 (C-2), 17.1 (C-27), 16.8 (C-7), 15.5 (C-21). FAB *m/z*: calculated for $C_{28}H_{46}NO_8$ ($M + H^+$) 524.3223, found 524.3218.

*2.2.2 3-O-[3-(2,2,2-trifluoroethoxy)-5-methoxybenzoyl]veracevine (**21**)*

To an ice-cooled solution of veracevine (**1**) (65 mg, 0.127 mmol) and 4-dimethylaminopyridine (5 mg) in dichloromethane + pyridine (0.5 ml of each) were added *N,N'*-dicyclohexylcarbodiimide (50 mg, 0.242 mmol) and 3-(2,2,2-trifluoroethoxy)-5-methoxybenzoic acid (58 mg, 0.232 mmol): the latter was prepared by etherification of methyl 5-hydroxy-3-methoxybenzoate by 2,2,2-trifluoroethyl *p*-toluenesulfonate and subsequent saponification of the resultant ester. The mixture was stirred at room temperature for 16 h, then diluted with methanol + acetone (0.1 + 0.2 ml) and the solution loaded directly onto a preparative TLC plate for purification to afford the title compound (47 mg, 50% yield) as a white solid, mp 123–125°C. [^{1}H]NMR (diagnostic peaks listed only) δ: 7.24 (1H, dd, J = 1.1 and 2.4, ArH), 7.16 (1H, dd, J = 1.1

and 2.4, ArH), 6.71 (1H, t, J = 2.4, ArH), 5.16 (1H, d, J = 4.0 Hz, H-3), 4.7 (broad s, OHs), 4.38 (2H, q, J = 8.1 Hz, CF_3CH_2O), 4.15 (1H, t, J = 3.2 Hz, H-16), 3.86 (s, 3H, CH_3O), 1.16 (3H, s, H-21), 1.11 (3H, d, J = 7.2 Hz, H-27), 1.04 (3H, s, H-19). [^{13}C]NMR δ: 166.6 (C = O), 160.9, 158.4, 132.1, (aryl carbons) 123.2 (q, J = 278 Hz, CF_3), 109.8, 107.8, 106.4 (aryl carbons), 105.0 (C-4), 94.7 (C-9), 81.8 (C-12), 80.4 (C-14), 76.2 (C-3), 75.8 (C-20), 72.0 (C-17), 70.9 (C-16), 66.0 (q, J = 36 Hz, CF_3CH_2O), 63.8 (C-22), 61.3 (C-26), 55.8 (CH_3O), 51.4 (C-18), 46.1 (C-5), 45.6 (C-10), 44.8 (C-8), 42.1 (C-11), 36.9 (C-13), 32.6 (C-1), 31.1 (C-15), 29.0 (C-23), 27.5 (C-25), 26.7 (C-6), 19.0 (C-19 and C-24), 18.3 (C-2), 17.2 (C-27), 16.9 (C-7), 15.6 (C-21).

*2.2.3 3-O-(3,5-Diiodobenzoyl)veracevine (**25**)*

To an ice-cooled solution of veracevine (**1**) (35 mg, 0.068 mmol) and 4-dimethylaminopyridine (8 mg) in dichloromethane + pyridine (0.5 + 0.1 ml) was added 3,5-diiodobenzoic acid (55 mg, 0.147 mmol) followed by *N*,*N*′-dicyclohexylcarbodiimide (30 mg, 0.147 mmol). The reaction mixture was stirred at room temperature for 16 h, then diluted with methanol + acetone (0.1 + 0.2 ml) and loaded directly onto a preparative TLC plate for purification. Yield: 29 mg (49%).

White solid, mp 174–175°C. [^{1}H]NMR (diagnostic peaks listed only) δ: 8.25 (3H, m, ArH), 5.16 (1H, d, J = 4.0 Hz, H-3), 4.80 (broad s, OHs), 4.15 (1H, t, J = 3.0 Hz, H-16), 1.16 (3H, s, H-21), 1.09 (3H, d, J = 7.0 Hz, H-27), 1.05 (3H, s, H-19). [^{13}C]NMR δ: 164.0 (C = O), 149.5, 137.6, 133.0, 94.4 (aryl carbons), 104.6 (C-4), 94.7 (C-9), 81.7 (C-12), 80.3 (C-14), 76.3 (C-3), 75.7 (C-20), 71.9 (C-17), 70.8 (C-16), 63.5 (C-22), 61.1 (C-26), 51.3 (C-18), 45.9 (C-5), 45.4 (C-10), 44.6 (C-8), 42.0 (C-11), 36.8 (C-13), 32.4 (C-1), 31.0 (C-15), 28.9 (C-23), 27.4 (C-25), 26.5 (C-6), 19.0 (C-19 and C-24), 18.3 (C-2), 17.1 (C-27), 16.9 (C-7), 15.5 (C-21).

*2.2.4 3-O-(3-Thienoyl)veracevine (**26**)*

This compound was prepared from **1** and 3-thiophenecarboxylic acid as described for **25** in 42% yield as a white solid, mp 128–130°C. [^{1}H]NMR (diagnostic peaks listed only) δ: 8.13 (1H, dd, J = 1.1 and 2.0 Hz, ArH), 7.43 (1H, dd, J = 1.1 and 4.2 Hz, ArH), 7.32 (1H, dd, J = 2.0 and 4.2 Hz, ArH), 5.11 (1H, d, J = 4.3 Hz, H-3), 4.70 (broad s, OHs), 4.15 (1H, t, J = 3.0 Hz, H-16), 1.15 (3H, s, H-21), 1.09 (3H, d, J = 7.0 Hz, H-27), 1.02 (3H, s, H-19). [^{13}C]NMR δ: 163.1 (C = O), 133.3, 132.4, 127.9 and 126.2 (aryl carbons), 105.0 (C-4, 94.5 (C-9), 81.7 (C-12), 80.3 (C-14), 75.7 (C-20), 75.5 (C-3), 72.0 (C-17), 70.9 (C-16), 63.5 (C-22), 61.2 (C-26), 51.3 (C-18), 46.1 (C-5), 45.5 (C-10), 44.7 (C-8), 42.1 (C-11), 36.9 (C-13), 32.5 (C-1), 31.1 (C-15), 29.0 (C-23), 27.5 (C-25), 26.7 (C-6), 19.0 (C-19 and C-24), 18.3 (C-2), 17.1 (C-27), 16.9 (C-7), 15.5 (C-21).

*2.2.5 3-O-[2-(5-Methoxythienoyl)]veracevine (**27**)*

This compound was prepared from **1** and 2-(5-methoxythiophene)carboxylic acid[20] as described above in 48% yield as a white solid, mp 171–172°C. [^{1}H]NMR (diagnostic peaks listed only) δ: 7.56 (1H, d, J = 4.2 Hz, ArH), 6.23 (1H, d, J = 4.2 Hz, ArH), 5.06 (1H, d, J = 4.2 Hz, H-3), 4.70 (broad s, OHs), 4.15 (1H, t, J = 3.0 Hz, H-16), 3.95 (3H, s, CH_3O), 1.15 (3H, s, H-21), 1.09 (3H, d, J = 7.0 Hz, H-27), 1.01 (3H, s, H-19). [^{13}C]NMR δ: 163.0 (C = 0), 143.3, 134.0, 117.9 and 105.8 (aryl carbons), 105.0 (C-4), 94.5 (C-9), 81.7 (C-12), 80.2 (C-14), 75.7 (C-20), 75.6 (C-3), 71.9 (C-17), 70.9 (C-16), 63.6 (C-22), 61.2 (C-26), 60.4 (CH_3O), 51.3 (C-18), 46.0 (C-5), 45.5 (C-10), 44.7 (C-8), 42.0 (C-11), 36.8 (C-13), 32.5 (C-1), 31.0 (C-15), 29.0 (C-23), 27.4 (C-25), 26.7 (C-6), 19.0 (C-19 and C-24), 18.3 (C-2), 17.1 (C-27), 16.9 (C-7), 15.5 (C-21).

2.3 Biological activity

2.3.1 Test solutions

Unless otherwise noted, 0.2 M stock solutions of the test compounds were prepared in methanol and stored below − 20°C in the dark.

2.3.2 Test organisms

All test species were from the collection of the Plant Protection Institute, Hungarian Academy of Sciences, Budapest.

2.3.2.1 Bacteria. The following species were used: *Agrobacterium radiobacter* (Beijerinck & vanDelden) Conn; *A tumefaciens* (Smith & Townsend) Conn; *Rhizobium trifolii* Dangeard; *Erwinia atroseptica* Dye; *E herbicola* (Löhnis) Dye; *E uredovora* Dye; *Pseudomonas fluorescens* Migula; *P phaseolicola* (Burkholder) Dowson; *P syringae* van Hall; *Xanthomonas alfalfae* (Riker, Jones & Davis) Dowson; *X malvacearum* (Erw Smith) Dowson; *X phaseoli* Burkholder; *X stewartii* (Erw Smith) Dowson; *Escherichia coli* (Migula) Castellani & Chalmers, *Corynebacterium betae* Keyworth; *C fascians* (Tilford) Dowson; *C flaccumfaciens* (Hedges) Dowson; *C michiganense* (EF Smith) Jensen; *C oortii* Collins & Jones, *Staphylococcus aureus* Rosenbach, *Micrococcus luteus* (Schroeter) Cohn, *Bacillus subtilis* (Ehrenberg) Cohn and *B thuringiensis* Berliner subsp *kurstaki*.

The bacteria were maintained on Nutrient Agar (Oxoid CM3) completed with vitamins (pyridoxine HCl, thiamine HCl, riboflavin and nicotinamide at 1.0, 10.0, 1.0 and 20.0 mg litre^{-1}, respectively). Bacterial suspensions for screening were prepared by washing cells with sterile tap water containing 0.3% peptone from slants of 20-h-old cultures grown at 21(± 1)°C. The optical density of the suspensions used as inoculum was adjusted to 0.2 (520 nm in 1 cm cuvette) by adding sterile distilled water to give 1–5 × 10^7 cell ml^{-1}.

2.3.2.2 Fungi. The following species were used: *Mucor racemosus* L; *Rhizopus stolonifer* (Ehrenberg) Lind; *Saccharomyces cerevisiae* Meyer ex Hansen; *Alternaria solani* (Ellis & Martin) Jones & Grout.; *Aspergillus niger* van Tiegh; *Botrytis allii* Munn; *Botrytis cinerea* Pers; *Cladosporium cucumerinum* Ellis & Arthur; *Colletotrichum coccodes* (Wallr) Hughes; *Glomerella cingulata* (Stoneman) Spaulding & Schrenk; *Fulvia fulva* Cooke; *Fusarium oxysporum* Schlecht; *F. graminearum* Schwabe; *Myrothecium roridum* Tode; *Penicillium oxalicum* Currie & Thom; *Pyricularia oryzae* Briosi & Cavara; *Septoria lycopersici* Spegazzini; *Thielaviopsis basicola* (Berk & Broome) Ferraris; *Trichothecium roseum* Link ex Fries; *Trichoderma harzianum* Rifai; *Verticillium alboatrum* Reinke & Berthold; *Schroeteria decaisneana* (Boudier) de Toni and *Ustilago maydis* (D C) Corda.

Saprophytic and facultative parasitic fungi were maintained on potato dextrose agar (Merck, Darmstadt, Germany) slants. For producing conidia, the strains were grown on malt agar slants (MEB and agar-agar, 17 and 11 g litre^{-1}, respectively) containing inorganic salts (KCl, $MgSO_4 \cdot 7H_2O$, $NaNO_3$, and $Fe(II)SO_4 \cdot 5H_2O$ at 0.574, 0.558, 1.30 and 0.01 g litre^{-1}, respectively), Na-β-glycerophosphate $\cdot 6H_2O$ (0.66 g litre^{-1}), citric acid (0.2 g litre^{-1}) and vitamins (pyridoxine $\cdot$ HCl, thiamine $\cdot$ HCl, riboflavin and nicotinamide at 1.0, 10.0, 1.0 and 20.0 mg litre^{-1}, respectively). Inocula were prepared by suspending conidia (10^5 cell ml^{-1}), sporidia or vegetative cells (10^4 cell ml^{-1}) in sterile distilled water containing glucose and Tween 40 (2.0 and 0.2 g litre^{-1}, respectively). The obligate parasite *Erysiphe graminis* DC f sp *tritici* Marchal was maintained on leaves of greenhouse-grown wheat (*Triticum aestivum* L cv Besostaya).

2.3.2.3 Oomycota. Two facultative parasites, *Pythium irregulare* Buism and *Phytophthora cryptogea* Pethyb & Laff were maintained on green pea agar (GPA). For both species, four-day-old colonies, grown on cellophane film on the surface of GPA plates, were gently triturated in green pea broth and these suspensions (50 mg mycelium ml^{-1}) were used as inoculum.

The obligate parasite *Plasmopara halstedii* (Farl) Berlese & de Toni race 1, was maintained on sunflower plants (*Helianthus annuus* L cv GK-70). All procedures involving production, inoculation and subsequent cultivation of infected seedlings were carried out as described previously.[21] Zoosporangia were obtained from systemically infected six- to eight-day-old plants incubated in a moist chamber for 18–20 h at 16($\pm$1)°C. The zoosporangia formed were washed off with ice-cold sterile distilled water and this suspension was filtered first through Perlon meshwork (No 500). The zoosporangia were collected on glass filters (Pyrex, G3) by gravitational filtration, then resuspended in ice-cold sterile double-distilled water and filtered again to eliminate any bacterial contaminant. The zoosporangia were then resuspended and their concentration was adjusted to 2.5×10^5 cell ml^{-1} using a haemocytometer. This standard suspension was used for screening tests and to produce zoo- and cystospores according to Virányi & Oros.[22]

2.3.2.4 Plants. For assessing the phytotoxicity of the compounds, the sunflower and wheat varieties were the same as those used for maintaining the obligate parasites.

2.3.3 Biological assays

2.3.3.1 Antibacterial tests. The culture medium used for testing consisted of agar, glucose, peptone, yeast extract, glycerol, Na-β-glycerophosphate $\cdot 6H_2O$, KH_2PO_4, $Na_2HPO_4 \cdot 7H_2O$, KCl, $MgSO_4$, $CaCl_2$, $FeSO_4 \cdot 5H_2O$, $CuSO_4 \cdot 5H_2O$, $MnSO_4 \cdot H_2O$, $Ni(NO_3)_2 \cdot 6H_2O$, and $CoCl_2 \cdot 6H_2O$ (11.0, 5.0, 5.0, 3.0, 2.0, 0.5, 0.5, 0.5, 0.25, 0.15, 0.15, 0.025, 0.005, 0.005, 0.0001 and 0.0001 g litre^{-1}, respectively). For determination of antibacterial activity, the appropriate amount of the test compound was mixed with the medium (10 ml) before pouring into Petri dishes (90 mm diameter). The agar plates were then inoculated with bacterial suspensions using a multipoint inoculator. The intensity of colony growth was evaluated after 24 h incubation at 21($\pm$1)°C using the scale: 0 = no growth, 1 = growth just visible, 2 = retarded growth, 3 = not visually distinguishable from the untreated control.

2.3.3.2 Antifungal tests. For the in-vitro assays, agar, malt and yeast extracts, Na-β-glycerophosphate $\cdot 6H_2O$, KH_2PO_4, $Na_2HPO_4 \cdot 7H_2O$, KCl, and $MgSO_4$ (11.0, 10.0, 1.0, 0.6, 0.25, 0.25, 0.15, 0.25 g litre^{-1}, respectively) were added to potato broth (1 litre). The broth was prepared from peeled potato tubers (200 g) cut into pieces (approx. 1 × 1 × 1 cm) and cooked in 1 litre of distilled water (20 min) then filtered through a Perlon meshwork (No 500) and the filtrate was then made up to 1 litre with distilled water. The medium was inoculated with 10^5 propagules of the fungus and a 5-mm-deep layer was dispensed into Petri dishes (90 mm diam.). Filter paper discs (5 mm diam.) impregnated with 10^{-6} mole of test compound were placed centrally on the agar plate (one disc per dish) and growth inhibition zones were measured after 48 h.

For in-vivo assay, cotyledons of wheat infected one day earlier with *E graminis* were sprayed to run-off with an aqueous solution of the test compound (50 μM) and the number of colonies was counted when sporulation of powdery mildew started on untreated control plants.

2.3.3.3 Anti-oomycete activity tests. For in-vitro assays, GPA was prepared by adding agar, glucose,

Trypton T, yeast extract, Na-β-glycerophosphate $\cdot 6H_2O$, KCl, and $MgSO_4$ (11.0, 10.0, 2.0, 0.5, 0.5, 0.25, 0.15 g litre^{-1}, respectively) to broth (1 litre) made from green peas. The broth was prepared from green peas (450 g) cooked in distilled water (1 litre; 20 min) then filtered through a Perlon meshwork (no. 500) and the filtrate was then made up to 1 litre with distilled water. The GPA medium was inoculated with *P irregulare* or *P cryptogea* mycelium suspension (1 ml per dish; see Section 2.3.2) and a 5-mm-deep layer of this culture medium was dispensed into Petri dishes (90 mm diam.). Filter paper discs (5 mm diam.) impregnated with 10^{-6} mole of test compound were then placed centrally on the agar plate (one disc per plate) and the diameter of growth inhibition zones was measured after 72 h.

Both young and well-established hyphae of *P halstedii* (SDM fungus) were examined.

Method A: Germlings of sunflower infected previously were treated by the whole-seedling immersion method[23] with aqueous solutions of the test compounds (0.5 ml of 2.0, 20, 100 or 200 μM) and then incubated for 18 h. They were then sown into soil (five per 0.5-kg pot) and grown in the greenhouse at 15-h light-days according to Virányi & Oros.[22] Occurrence of damping-off and the number of plants bearing chlorotic leaves were recorded when typical symptoms of SDM disease on untreated control plants developed. The survival of the thallus in symptomless individuals was examined by making sections for microscopic survey according to Virányi.[24]

Method B: Hypocotyl segments (1 cm long) cut from eight-day-old infected sunflower seedlings were immersed in an aqueous solution of the test compound (1.0 mM; 0.5 ml for each segment) and incubated for 16 h at 16($\pm$1)°C, after which the segments were transferred into Petri dishes lined with wet filter paper and incubated in a moist chamber at 21($\pm$1)°C to induce zoosporangium formation. The development of zoosporangiophores was recorded after 24 and 48 h.

To evaluate the effect of the test compounds on the asexual spores of *P halstedii*, the following parameters were recorded: viability and germination of zoosporangia, viability of zoospores (motion and plasmalemma semipermeability) and cystospore germination. All these events were observed microscopically in suspensions of spores (2.5×10^5 cells ml^{-1}) mixed with solutions (1 + 1 by volume) of the compounds at appropriate concentrations with ten-fold serial dilutions and incubating them at 16($\pm$1)°C. The lethal effect of test compounds on zoosporangia was determined by adding an aqueous solution of Rose Bengal (0.1 mg ml^{-1}) to a sample (1 + 2, by volume) of the cell suspension. Non-viable zoosporangia stained deep purple while dormant (viable) ones remained unstained. The effect on motility and plasmalemma function was examined by incubating freshly prepared zoospores with the test compounds for 15 min and recording the respective doses at which cell motion stopped or cell disruption occurred, giving minimum inhibitory concentration (MIC) values for either function. To assess the effect of the compounds on cystospores the minimum dose totally inhibiting germination was recorded after an incubation for 18 h.

2.3.3.4 Assessment of phytotoxicity. The number of injured and/or abnormally developed wheat and sunflower plants was recorded and the type of alteration noted up to 21 days after treatment.

2.3.3.5 Data analysis. Assays were replicated at least three times. Fisher's probe was applied for testing least significant difference (LSD) values of biological response data. Multiple regression was carried out according to Sváb.[25]

3 RESULTS

3.1 Antibacterial activity

The antibacterial spectrum of selected test compounds at 10 μM dose is presented in Table 2. Veracevine (**1**) and naturally occurring sabadilla components **2**–**5** exhibited similar activity profiles although their antibacterial spectrum was restricted to *Erwinia* and *Corynebacterium* species. Of the semisynthetic esters, the cyclopropylcarboxylate **9**, pivaloate **10**, and tigloate **12**, being the geometric isomer of **3**, showed notable activity. Compound **11**, the saturated analogue of **3** and **12**, was devoid of antibacterial activity. Oleate **13** inhibited only *E atroseptica*, and then only slightly. Of the aromatic series, only compound **22** showed significantly higher activity than the natural ester veratridine (**3**). Conversion of the characteristic hemiacetal ring of **1** into the ketol cevagenine (**31**) (Fig 1) did not affect the antibacterial activity. While cevine (**6**), the 3α-epimer of the alkamine **1**, showed no substantial effect against any bacterium species at 10 μM, germine (**35**), another veracevine isomer in which the 3β-hydroxy group is retained but other hydroxy groups are arranged differently on the cevane skeleton (see Fig 1), was equipotent to **1**.

The activity of the most potent compounds **22** and **31** against *Erwinia* species ranked between that of tridemorph and dodine (Table 2), two fungicides with known antibacterial side effects.[26,27] At the discriminatory dose used, the antibacterial activities of the test alkaloids and of dodine and metalaxyl were significantly lower than those of polymyxin B and streptomycin, two broad-spectrum antibacterial antibiotics.

3.2 Antifungal activity

The antifungal spectra of veracevine (**1**) and of those of its derivatives showing detectable activity against more than one of the 23 species tested at 1 μmole

Table 2. Antibacterial effect of ceveratrum alkaloids and reference compounds *in vitro* at 10 μM

Species[b]	*Colony growth response*[a] *to treatment with*																	
	Alkaloids[c]														*Fungicides*[d]		*Antibiotics*[e]	
	1	**2**	**3**	**4**	**5**	**6**	**8**	**9**	**10**	**12**	**13**	**22**	**31**	**35**	**TDM**	**DOD**	**PMB**	**STR**
Gram negatives																		
A radiobacter [K-84]	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	0	2
A tumefaciens [O]	3	3	3	3	3	3	3	3	3	3	3	3	3	3	2	3	0	1
A tumefaciens [C-54]	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	0	1
R trifolii	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	2	0	2
E. atroseptica	1	3	2	1	1	2	3	1	1	1	3	2	1	2	2	3	0	1
E. herbicola	2	2	2	2	2	3	3	2	2	2	3	3	2	2	3	2	0	0
E uredovora	2	2	2	2	2	3	3	2	2	2	2	2	2	3	3	3	2	0
P fluorescens	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	1	0	0
P phaseolicola	2	3	3	2	2	3	3	2	2	2	3	3	2	3	3	3	2	0
P syringae	3	3	3	3	3	3	3	3	3	3	3	2	3	3	3	3	0	2
X alfalfae	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	2	2	0
X malvacearum	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	2	2	1
X phaseoli	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	2	0	0
X stewartii	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	1	0	0
E coli	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	0	0
Gram positives																		
C betae	3	3	2	3	3	3	3	3	2	3	3	2	2	3	2	2	3	0
C fascians	2	3	2	2	2	3	2	2	2	2	3	2	3	3	3	0	3	0
C flaccumfaciens	3	3	3	3	3	3	3	3	3	3	3	1	2	3	2	0	1	0
C michiganense	2	3	2	2	2	3	2	2	2	2	3	1	2	2	2	0	3	0
C oortii	3	3	2	3	3	3	3	3	3	3	3	2	2	3	3	0	3	0
M luteus	2	2	2	2	2	3	2	2	2	2	3	2	2	2	1	1	1	0
S aureus	3	3	3	3	3	3	3	3	3	3	3	3	3	3	2	3	2	0
B subtilis	3	3	3	3	3	3	3	3	3	3	3	2	3	3	2	1	3	0
B thuringiensis	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	1	0

[a] The intensity of colony growth was evaluated using the grade scale: 0 = no growth, 1 = growth just visible, 2 = apparent retarded growth, 3 = not visually distinguishable from control.
[b] For full names of organisms see Section 2.3.2.
[c] Compounds listed in Table 1 but not included here were inactive or inhibited the growth of not more than one species.
[d] TDM = tridemorph, DOD = dodine.
[e] PMB = polymyxin B, STR = streptomycin.

dose in a diffusion assay *in vitro* are presented in Table 3. In general, the activity of natural ceveratrum alkaloids **1**–**5** is limited to *F graminearum* and *G cingulata*, both species belonging to Hypocreales. The activity profile of germine (**35**) was similar. In contrast, the reference fungicides displayed a broader spectrum of activity and their activity profile was different: tridemorph was active only against asco- and basidiomycetes, the antibiotics blasticidin-S and nystatin affected all fungi tested, while dodine and metconazole inhibited all test species. Of the two anti-oomycete fungicides, dimethomorph exhibited weak activity against some fungi from other classes, while metalaxyl was completely inactive, except against Oomycetes.

In general, various modifications of the acyl substituents at the 3-OH group of **1** or the steroid skeleton, such as in compounds **31**–**34**, did not improve the antifungal activity of the parent alkamine (data not shown). Oleate **13**, however, exhibited some activity against *C coccodes* and *F graminearum* comparable to that of tridemorph and blasticidin-S (Table 3).

In diffusion assays, alkaloids **1**–**34** had no effect on *P irregulare* and *P cryptogea* at a 1 μmole dose (Table 3). Dodine, metalaxyl and metconazole were active against both species, while dimethomorph and blasticidin-S affected only the latter. Tridemorph and nystatin were inactive in this assay.

At 50 μM dose none of the alkaloids **1**–**34** inhibited powdery mildew on wheat, against which metconazole and tridemorph gave $> 95\%$ control in comparative treatments.

3.3 Activity against sunflower downy mildew

The sensitivity of the SDM pathogen to the test compounds varied considerably during its vegetative life cycle (Table 4). Responses of the biotrophic thallus of *P halstedii* to sabadilla components and selected derivatives of veracevine (**1**) were examined.

Table 3. Selective toxicity of representative ceveratrum alkaloids and reference compounds *in vitro* against fungi of various taxonomic position.

Species[b]	*Diameter of inhibitory zones*[a] (*mm*)															
	Alkaloids[c]								*Fungicides*[d]					*Antibiotics*[e]		
	1	**2**	**3**	**4**	**5**	**12**	**13**	**35**	**DOD**	**TDM**	**MCA**	**DMM**	**MLX**	**NYS**	**BLS**	**STR**
Oomycota																
P irregulare	0	0	0	0	0	0	0	0	12	0	31	0	67	0	0	0
P cryptogea	0	0	0	0	0	0	0	0	10	0	20	57	54	0	21	8
Zygomycota																
M racemosus	0	0	0	0	0	0	0	0	11	0	48	0	0	10	0	0
R nigricans	0	0	0	0	0	0	0	0	13	0	47	0	0	27	41	0
Ascomycota																
S cerevisiae	0	0	0	0	0	0	0	0	10	20	17	6	0	33	44	0
A solani	0	0	0	0	0	0	0	0	20	18	56	0	0	43	25	23
A niger	0	0	0	0	0	0	0	0	10	16	56	5	0	19	26	0
B allii	0	0	0	0	0	0	0	0	16	21	55	7	0	41	21	0
B cinerea	0	0	0	0	0	0	0	0	10	22	53	6	0	29	12	0
C cucumerinum	0	0	0	0	0	0	0	0	14	11	65	0	0	23	0	0
C coccodes	0	0	0	0	0	0	15	0	10	0	53	0	0	29	13	0
G cingulata	7	6	7	7	7	7	8	0	9	6	45	0	0	18	14	0
F fulva	0	0	0	0	0	0	0	0	32	90	90	0	0	63	41	0
F oxysporum	0	0	0	0	0	0	0	0	13	0	53	0	0	17	11	0
F graminearum	8	7	8	8	7	8	15	7	21	6	55	0	0	29	11	0
M roridum	0	0	0	0	0	0	0	0	12	6	51	0	0	16	0	0
P oxalicum	0	0	0	0	0	0	0	0	11	0	42	0	0	24	13	0
P oryzae	0	0	0	0	0	0	0	0	15	35	54	8	0	42	33	0
S lycopersici	0	0	0	0	0	0	0	0	21	90	45	0	0	49	31	0
T basicola	0	0	0	0	0	0	0	0	15	20	67	7	0	8	43	0
T harzianum	0	0	0	0	0	0	0	0	10	0	50	0	0	27	20	0
T roseum	0	0	0	0	0	0	0	0	14	18	53	0	0	7	0	0
V albo-atrum	0	0	0	0	0	0	0	0	13	7	68	0	0	33	8	0
Basidiomycota																
S decaisneana	0	0	0	0	0	0	0	0	10	21	47	0	0	37	0	0
U maydis	0	0	0	0	0	0	0	0	14	34	15	7	0	35	27	6

[a] Results represent the mean of quadruplicate measurements, $LSD_{5\%} = 4.5$ and 6.0 mm within the compounds and species, respectively.
[b] For full names of organisms see Section 2.3.2.
[c] For structures, see Table 1 and Fig. 1.
[d] DOD = dodine, TDM = tridemorph, DMM = dimethomorph, MLX = metalaxyl, MCA = metconazole.
[e] NYS = nystatin, BLS = blasticidin-S, STR = streptomycin.

The alkaloids assayed did not particularly affect penetration and colonisation, the initial steps of the biotrophic stages of the SDM pathogen (Method A), when plants were treated after infection with 20 μM solutions in the germling stage (data not shown). However, they altered the pattern of the symptomatic picture of the SDM.

Similarly to metalaxyl, veracevine (**1**), but not its 3α epimer **6**, significantly decreased the frequency of damping-off (Table 4). Aromatic esters **17**, **18** and **27** were as potent as the parent alkamine **1**, while other esters were less active.

Leaf chlorosis, the most characteristic symptom of SDM syndrome, was absent even in those alkaloid-treated plants where the entire epicotyl on the lower part of the first internode was colonised. The microscopic survey revealed that even a 200-μM treatment of the germlings post-infection (Method A) failed to eliminate parasitic hyphae from the plants. Compounds with an unsubstituted 3-OH (**1**, **6** and **35**) and the other derivatives tested (see Table 4) significantly prevented the occurrence of leaf chlorosis. Nevertheless, zoosporangium formation could be induced in all parts of these symptomless plants where the pathogen was present (as evidenced by microscopic examination) (Method A; data not shown). Similarly, zoosporangia were formed on detached hypocotyl segments treated with the alkaloids at 200 μM (Method B; data not shown). When spores formed on sections of treated plants or hypocotyl segments were used to generate a new vegetative life cycle, the ratio of systemically colonised plants was >80% in each case, so that the viability of the thallus in host tissues treated with alkaloids was not significantly affected. These observations indicate that the ceveratrum alkaloids have no effect on

Table 4. Toxicity of ceveratrum alkaloids and their semisynthetic derivatives to various developmental forms of sunflower downy mildew fungus

Compounds[a]	*Inhibition of Plasmopara halstedii in*							
	Host-dependent (biotrophic) stages Percentage ratio of plants with[b]			*Host-independent (abiotrophic) stages Minimum inhibitory concentrations (μM)*[c]				
	Latent infection	*Damping off*	*Leaf chlorosis*	*Zoosporangium*		*Zoospores*		*Cystospores Germination*
				Survival	*Germination*	*Membrane*	*Motility*	
Sabadilla components								
1	67	7	27	>1000	100	100	10	100
2	80	20	0	>1000	100	100	0.1	10
3	80	20	0	>1000	100	100	0.1	10
4	47	53	0	>1000	100	100	100	0.1
5	33	40	27	>1000	100	100	100	10
Semisynthetic derivatives								
6	67	33	0	>1000	100	100	1	10
7	0	32	67	>1000	100	100	100	100
8	nt	nt	nt	100	100	100	10	1
9	nt	nt	nt	100	100	100	10	1
10	60	33	7	>1000	100	100	10	1
11	47	53	0	>1000	100	100	0.1	0.1
12	nt	nt	nt	100	100	100	10	1
13	87	13	0	100	100	1	0.01	1
14	nt	nt	nt	1000	100	10	10	10
15	nt	nt	nt	1000	100	10	10	0.001
16	nt	nt	nt	100	100	10	10	1
17	nt	nt	nt	1000	10	10	10	10
18	93	7	0	1000	100	10	0.1	1
19	93	7	0	100	10	1	0.1	0.001
20	nt	nt	nt	100	10	1	1	0.1
21	87	13	0	100	10	10	0.1	0.1
22	nt	nt	nt	100	10	1	0.1	0.1
23	nt	nt	nt	100	100	100	10	1
24	nt	nt	nt	10	10	0.01	0.01	0.1
25	60	27	13	>1000	100	100	1	1
26	87	13	0	1000	100	10	1	1
27	93	0	7	100	100	10	0.1	1
28	nt	nt	nt	100	100	10	5	1
29	nt	nt	nt	100	10	10	1	0.1
30	nt	nt	nt	100	100	100	10	1
Other compounds								
31	87	13	0	1000	100	100	1	1
32	nt	nt	nt	100	100	100	10	1
33	53	33	13	>1000	100	100	100	10
34	nt	nt	nt	100	100	10	10	1
35	57	20	23	>1000	100	100	100	10
Reference fungicides								
DMM	13	33	53	1000	100	100	10	10
MLX	73	0	27	>1000	>1000	>1000	>1000	1000
TDM	0	30	70	>1000	1000	100	10	10
$CuSO_4$	0	35	65	>1000	>1000	1	0.1	100
Control								
Solvent	5	37	58	*no effect*				

[a] For specific structures of alkaloids **1–35**, see Table 1 and Fig 1; DMM = dimethomorph, MLX = metalaxyl, TDM = tridemorph.
[b] The results of responses of the biotrophic thallus measured at 20 μM dose represent the mean of triplicate treatments (Method A), $LSD_{5\%}$ = 11% within the compounds; nt = not tested.
[c] Determined from three series and five replicates in each.

zoosporangiogenesis. Among the reference fungicides, only metalaxyl was active against the thallus (Table 4), and none of them influenced the symptomatic pattern of SDM syndrome.

The activity of test compounds on various abiotrophic developmental forms of *P halstedii* varied greatly (Table 4). At 1 mM concentration, the highest test dose, veracevine (**1**) and its natural esters **2–5** did

not completely inhibit the formation of zoospores in freshly prepared zoosporangia after an 18 h incubation. Although the majority of the zoosporangia were killed, 15–30% of them always survived the treatment and remained resting, as was also seen for reference fungicides. Most of the semi-synthetic alkaloids, however, were effective in killing zoosporangia, aromatic esters, in general, being more potent than aliphatic ones. Interestingly, the angeloyl ester **3** was at least ten-fold less active than the isomeric tigloyl derivative **12**. Germination, ie the release of zoospores, was similarly more sensitive to aromatic ester derivatives, and these surpassed the reference compounds against this stage of the fungus.

Of the microscopically detectable events, the inhibition of motion and plasmalemma semipermeability were used to characterise the effect of the compounds on zoospores. The cevertrum alkaloids affected these cell functions in a distinct manner. The destruction of zoospores proceeded in three steps: initially, cell motion ceased (5–30 s), then their volume increased (15–60 s) followed by a further rapid swelling (10–30 s) that led to bursting of the cell.

In destroying the integrity of plasmalemma, the semi-synthetic aromatic esters were more effective than their natural counterparts **4** and **5** and aliphatic esters **2**, **3** and **7**–**12**. The MIC value of 3,4-dichlorobenzoyl ester **24**, the most potent compound in this respect, was 10^4-fold lower than that of veratridine (0.01 and 100 μM, respectively). The oleoyl ester (**13**) was also highly active (MIC = 1 μM). Simple ester derivatives of veracevine inhibited the motility apparatus of zoospores more effectively than the parent alkamine **1**, except vanillate **4** and veratrate **5**. Esters **13** and **24** were again the most potent alkaloids for this event (MIC = 0.01 μM for both compounds). Saturation of the C=C double bond of cevadine (**3**), leading to diastereomers **11**, did not influence activity, while the geometric isomer **12** was 100-times less effective. Interestingly, veracevine (**1**) was less active than cevine (**6**), its 3α epimer. Modifications of the functionalities of the cevane skeleton of cevadine (compound **32**) or veratridine (compounds **33** and **34**) resulted in slight variations in the antizoospore effect.

In inhibition of cystospore germination, all ester alkaloids were generally more effective than veracevine (**1**). In this assay, aromatic esters were again highly active: the MIC value of the most potent compounds **15** and **19** was 0.001 μM in each case. Cevadine (**3**) had a MIC value of 10 μM which is 100-fold less than that of its saturated counterpart **11**. Here again, most of the cevertrum alkaloids were more active than the commercial fungicides used.

3.4 Effect of alkaloids on host plants

At 2 μM concentration none of the alkaloids **1**–**34** was phytotoxic to sunflowers. Treatments with 20 μM solutions of cevertrum alkaloids **8**, **18**, **19**, **27** and **33**, however, caused abnormal development of some of the plants. With the exception of cevagenine (**31**), all alkaloids affected the development of plants at doses of 100 μM or higher. The typical symptoms of injured plants were twisted stems and spear-like leaves with thickened sheets and irregular veination; the formation of parenchyma was also depressed regardless of whether the pathogen was present or not.

None of the test compounds caused phytotoxic symptoms on wheat.

4 DISCUSSION

Plants of the lily family are well known both for their poisonous properties and also for their medicinal value. Powdered rhizomes of the white hellebore (*Veratrum album* L), indigenous in Europe and Asia, and the green hellebore or Indian poke (*Veratrum viride* Ait), growing in the eastern part of North America, were used to cure herpes, toothache, rheumatism and catarrh. Drugs from these plants have also been important hypotensive agents. Insecticidal preparations from the powdered seeds of *S officinale*, indigenous in Mexico, Central America and the northern region of South America, have been used for centuries. Sabadilla powder is in current use against thrips in citrus and avocado due to its low persistence and compatibility with beneficial insects.[2,3] To date, more than 400 related natural and synthetic alkaloids have been described.[5,15] Although the allelopathic roles of plant alkaloids, in general, have been hypothesised,[12,13] the antimicrobial properties of the cevertrum group of *Veratrum* alkaloids have received scant attention.

Our present study, involving a broad range of phylogenetically different microbes, has revealed that the antimicrobial activity of sabadilla components **1**–**5** against bacteria and fungi is weaker than that of synthetic fungicides and antibiotics used as reference compounds. These alkaloids have a narrow activity spectrum affecting species of *Erwinia*, *Corynebacterium* (Table 2), *Fusarium* and *Glomerella* (Table 3) species. Variations in the R group (compounds **7**–**30**) or in the cevane skeleton (compounds **6**, **31**–**35**) resulted in no essential change in their antimicrobial spectrum of activity.

Activity of cevertrum alkaloids **1**–**35** against *P halstedii* was apparently dependent on the stage of development of the pathogen (Table 4). While there was no substantial difference between the most efficacious alkaloids (eg compounds **13**, **27** and **31**) and metalaxyl against biotrophic forms, against asexual spores the alkaloids, in general, proved to be 10–10^3 times more active than the reference fungicides. The most active alkaloids were at least as effective in controlling SDM as tridemorph and metalaxyl. These two commercial fungicides interrupt the ontogenesis of *P halstedii* only at a single developmental stage, eg

metalaxyl inhibits the parasiting hypha while tridemorph acts on the mobile zoospores. In fact, in mixtures they show excellent synergistic activity against SDM but the resting zoosporangia remain untouched.[28] In this respect, cevagenine (**31**) and the semi-synthetic esters **13**, **18**, **19** and **27** are noteworthy because they affect *P halstedii* at several points of the life cycle, including resting zoosporangia. Moreover, their activity against invading thalluses approached that of the above-mentioned synergistic mixture.

The ceveratrum alkaloids had no effect on penetration, colonisation and zoosporangium production of *P halstedii*. They did, however, suppress the progression of leaf chlorosis, suggesting that treatments at the germling stage have lasting influence on plant–microbe interaction in this host–parasite system. This assumption is supported by the fact that curative doses of cevacine (**2**) and cevadine (**3**) neither eradicated the parasiting thallus nor inhibited the subsequent ontogenetic steps of the fungus in tissues detached from treated plants, and the zoosporangia formed on such tissue were as vigorous as those from untreated plants. The semi-synthetic esters **13**, **18**, **19**, **21** and **27**, as well as the ketol **31**, with a *trans* A/B ring system lacking the hemiacetal oxygen bridge, caused similar alterations in the pattern of symptoms. The antiperonospora effect of these compounds was commensurable with those of metalaxyl and copper sulfate (Table 4).

The ability of the natural alkaloids **1**–**5** to penetrate cell walls may be a factor of their developmental stage-selective effect as the compounds inhibit wall-less zoospores more efficiently than the other, cell-wall-bearing ontogenetic forms. They were 10 to 10^4 times more effective against cystospores than against zoosporangia, which can also be related to differences in the cell wall composition of target cells. The nature of the acyl group influences the ability of the parent alkamine **1** to penetrate the cell wall, as indicated by qualitative changes in the activity against cell-wall-bearing spores. Staining of zoosporangia with Bengal Rose revealed that zoosporogenesis was inhibited shortly before or during the opening of the operculum. Distinctly from the other alkaloids and reference compounds, aliphatic esters **8** and **9**, as well as aromatic derivatives **15**, **19**–**23** and **27**–**30** effectively inhibited earlier stages of zoosporogenesis as seen by microscopic observation, indicating that these compounds could penetrate the cell wall of zoosporangia. Moreover, these derivatives inhibited cystospores at 10 to 10^4 times lower concentrations than the acetate ester, cevacine (**3**). When the 3-OH group was converted to the methyl ether (compound **7**) the antimicrobial activity drastically decreased.

The test compounds affected zoospore functions distinctly. Inhibition of the motility apparatus occurred after a concentration-dependent lag period which was substantially longer for tridemorph than for the ceveratrum alkaloids at comparative doses. The swelling dynamics, reflecting changes in plasmalemma semipermeability, showed that the zoospores were able to compensate the alkaloid-induced upset of osmotic balance by excreting water actively for a limited time, after which a rapid volume increases led to cell burst. This phenomenon could not be observed with tridemorph, where swelling proceeded without a lag-phase. The mode of action of the test alkaloids on the zoospores is apparently different from that of tridemorph.

The specific action of the ceveratrum alkaloids on the *P halstedii* cell membrane is unlikely to be a result of simple physicochemical interactions with membrane lipids because there was no correlation ($R < 0.5$) between their lipophilicity and membrane-destroying activities. The relationship between the structure and/or physicochemical properties, lipophilicity in particular, and the antimicrobial activity for this set of compounds is not apparent even when the results of assays with the cell-wall-less developmental stage of *P halstedii* are considered. Although the role of sterols in the physiology of *P halstedii* has not been studied, the observed rapid action of the alkaloids, especially pronounced against zoospores, excludes sterol biosynthesis as a potential target for these compounds in the abiotrophic developmental stages of the pathogen. The exact antimicrobial mode of action of the ceveratrum alkaloids requires further studies.

Modifications of the acyl chain had dissimilar effects on the insecticidal and antimicrobial activities. For example, while the natural esters, eg cevadine (**3**) and veratridine (**5**), are among the most potent insecticides, they are not good antimicrobial agents. The aliphatic oleoyl ester (**13**) and the aromatic 3,4-dichlorobenzoyl ester **24** had low insecticidal activity but affected *P halstedii* through all its developmental stages. However, the 3,5-dimethoxybenzoyl ester **18** had substantially improved insecticidal and antiperonospora activity with regard to the natural ester **5**. The toxicity of these three semi-synthetic alkaloids to sunflower was negligible.

In summary, neither the natural nor the semi-synthetic ceveratrum alkaloids examined in this study showed notable antibacterial effects in comparison with reference substances. In general, these compounds acted on a limited number of real fungi and the best derivative, the oleoyl ester **13**, could be ranked between dodine and tridemorph. Characteristically, most of the ester alkaloids **3**–**34** and cevagenine (**31**) exhibited remarkable but developmental stage-dependent antiperonospora activity disrupting the life cycle of *P halstedii* in both host-dependent and host-independent stages. Moreover, the ceveratrum alkaloids altered the pattern of the symptomatic picture of SDM. These are advantages compared to reference fungicides with different modes of action that affect the parasite only at separate, but single, ontogenetic stages and do not influence the

symptomatic pattern of SDM syndrome. Structural modifications of sabadilla alkaloids lead to compounds with improved antiperonospora activity comparable to that of the commercial fungicides tridemorph and metalaxyl. Thus, our study establishes the ceveratrum alkaloids as novel antifungal agents which are believed to act by disturbing membrane integrity.

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